

Irrigation to Maximize Vaccine Antigen Production in Genetically Modified Tobacco

G. Stevens,* E. Vories, M. Mulesky, M. Rhine, and D. Dunn

ABSTRACT

A protective antigen (PA) gene from *Bacillus anthracis* Cohn has been inserted into tobacco (*Nicotiana tabacum* L.) chloroplasts to produce an anthrax vaccine. The PA protein is the primary immunogen of human vaccines for anthrax disease. The objective of this study was to determine the optimum soil water content for producing antigen vaccine in tobacco leaves. High leaf antigen concentration is important in vaccine manufacturing. Protective antigen tobacco, transformed from 'Petite Havana SRI', was transplanted to native soil (fine-loamy, mixed, thermic Typic Argiudolls) in a greenhouse floor. Treatments consisted of soil water potential (Φ) irrigation thresholds (-20 , -34 , -48 , and -62 kPa) based on sensors buried 10 cm in soil. Since plant growth is normally stimulated when soil moisture is readily available, we hypothesized that soil moisture might dilute leaf PA content. Results showed that increasing biomass with optimum irrigation did not dilute PA in leaves. Using ELISA quantification, -34 kPa irrigation threshold averaged $529 \mu\text{g PA kg}^{-1}$ leaf fresh weight for two ratoon harvests. Leaves from drier irrigation treatments did not have significantly higher PA content or biomass. Tobacco grown with more frequent irrigations using -20 kPa Φ threshold averaged less biomass and produced leaves with significantly lower antigen. In a separate experiment, gravimetric soil water use was compared between non-transgenic Petite Havana and PA plants at button growth stage. Genetic engineering tobacco for PA caused reduced leaf area in plants. Transpiration was significantly higher with nontransgenic plants, indicating that lower irrigation rates may be needed for antigen tobacco.

TOBACCO has been genetically engineered to produce antibodies, biopharmaceuticals, and vaccines (Ohlrogge and Chrispeels, 1994). Growing plant-made pharmaceutical (PMP) tobacco in greenhouses is one option for increasing the supply of anthrax vaccine in the USA. Anthrax is an animal disease caused by *Bacillus anthracis*, a bacterium that forms spores. Humans can contract the disease from exposure to an infected animal or to animal products. Persons most likely to become infected are butchers, farmers, sheepherders, woolhandlers, and veterinarians. In 2001, anthrax was spread to people by terrorists who sent letters containing spores through the U.S. postal system (Day, 2003). Currently, anthrax vaccine is available in the USA to immunize people in high-risk jobs such as laboratory workers processing clinical samples and military personnel serving overseas. However, sufficient anthrax vaccine is not available to treat the general U.S. civilian population (Centers for Disease Control and Prevention, 2002).

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Petite Havana tobacco was transformed by Henry Daniell at the University of Central Florida to produce a recombinant PA which can be used to manufacture anthrax vaccine. Koya et al. (2005) reported on mice immunization research with tobacco chloroplast-derived anthrax PA vaccine. Protective antigen (83 kDa) is one of three proteins collectively known as the anthrax toxin which causes sickness or death. The other two proteins, lethal factor (LF, 90 kDa) and edema factor (EF, 90 kDa), have enzymatic functions but require PA to be biologically active (Williamson et al., 2005). Combined PA and LF cause death when injected intravenously into animals. Edema factor causes edema of the skin when injected. Protective antigen, the receptor-binding component of the toxin, is responsible for transport of the other two factors. A procedure patented by Chlorogen, Inc., was used to insert the PA gene from *B. anthracis* into the chloroplast genome of tobacco. Plant production of PA for vaccine is cleaner and safer than the current method using a cell-free toxigenic, non-encapsulated strain of *B. anthracis*. In rare cases, the current method has resulted in trace amounts of EF and LF in the filtrate, which can be toxic.

In this study, PA tobacco was used as a candidate model for managing irrigation in future antigen vaccine tobacco lines produced by chloroplast transformation. The transformation method used by Dr. Daniell to make PA tobacco directs integration of the transgene to the inverted repeated region on plant chloroplasts by homologous recombination. The precise insertion in chloroplast DNA eliminates the position effect which can be a problem in plants that have had nuclear transformation. Therefore, extrapolating results from soil and water experiments with PA tobacco to other chloroplast-based antigen tobacco lines is more reasonable than it would have been if we were comparing nuclear transformed tobacco lines.

A high target protein-to-biomass ratio in tobacco leaves allows for a reduction in the size of upstream processing equipment and costs in pharmaceutical production (Oishi et al., 2004). Upstream processing of green biomass usually includes screw pressing and filtration. The initial concentration of target protein in tobacco leaves also affects downstream processing costs in terms of volume reduction steps and ratios of transgene protein to host and environmental contaminating molecules. Resins are used for purification in a variety of configurations such as standard downflow system, radial chromatography, or streamline expanded-bed technology.

Optimum tobacco biomass yields require adequate sunlight, water, nutrients, and carbon dioxide. The effect of each of these factors on antigen concentration in

Abbreviations: Φ , soil water potential; PA, protective antigen; PAR, photosynthetically active radiation; PMP, plant-made pharmaceutical.

tobacco leaves is not known. We hypothesized that providing optimum soil water conditions with irrigation might dilute leaf antigen concentration in genetically modified tobacco by increasing plant biomass. Research with nutrients in plant leaves has shown that dilution sometimes occurs in rapid growth environments. Mills and Jones (1996) reported that increased soil moisture tends to lower the concentration of nutrient elements in tissue since plant growth is normally stimulated when soil moisture is readily available. The resulting dry matter increase dilutes the elemental content in the plant even though ideal soil moisture conditions also increase element availability.

The goal of an antigen tobacco production system is to maximize antigen expression and green leaf biomass in contrast to production of conventional tobacco, which is to produce tobacco leaves to satisfy consumer demand for cigarettes, cigars, or chewing tobacco. In conventional tobacco, Atkinson et al. (1971) evaluated burley plant populations ranging from 16,550 to 33,100 plants ha⁻¹. The highest plant density (33,100 plant ha⁻¹) produced significantly greater leaf yields than lower plant populations. However, income above added labor costs did not increase significantly when plant populations were increased above 19,860 plants ha⁻¹. Peedin et al. (1979) found that average market price of cured leaves was not influenced by plant spacing, while alkaloid and N concentrations were inversely related to population. In 2003 and 2004, M. Mulesky (unpublished data) found that PMP protein yields were optimal with dense transgenic plant populations (79,040 to 107,593 plants ha⁻¹) on narrow rows and multiple cuttings in a season. Ratoon cropping was used similar to alfalfa hay production. In conventional tobacco production, flower buds are *topped* as they form and leaves and stems are allowed to continue growing (McNess, 2001). Because of the high plant densities used to maximize antigen vaccine production, it is likely that soil water management will not be the same for transgenic vaccine tobacco and conventional tobacco production.

No research has been reported in the literature concerning transgenic antigen tobacco leaf protein or biomass response to irrigation. Field irrigation research in Kentucky and Maryland showed that conventional tobacco yield response to watering varies from year to year based on rainfall (Atkinson et al., 1971; Brown and Street, 1972). To optimize tobacco irrigation management, tobacco plant stage of growth and soil water-holding capacity should be considered (Reed et al., 1994). Terry and Terrill (1971) found that postplanting irrigation increased transplant survival 30% as compared with no irrigation. Maw et al. (1997) found greater leaf area and dry weight under an irrigation trigger level of -25 kPa Φ as compared with -100 kPa.

Excessive soil water can produce a poor root system and reduce yield in conventional tobacco (Hunt et al., 1981). How excessive soil water impacts antigen vaccine tobacco is not known. Huck (1970) found that conventional tobacco roots did not survive low oxygen conditions for more than 48 h. Georgia extension spe-

cialists recommend growing tobacco on high and wide row ridges to reduce the negative effects of excessive water from large rainfall events (Moore and Tyson, 1998).

The objective of a genetically modified tobacco study reported herein was to determine the optimum soil water content to produce the highest yields of leaf biomass and antigen content in leaves for manufacturing antigen vaccine.

METHODS AND MATERIALS

Two irrigation experiments were conducted in a glass greenhouse at the University of Missouri-Delta Research Center located in Portageville, MO (36°N, 90°W). The greenhouse is a Biosafety Level 1 facility. The USDA Animal and Plant Health Inspection Service (APHIS) issued a permit to grow regulated genetically modified tobacco for bioterrorism vaccines at this greenhouse. Field planting of vaccine tobacco was not included in the permit.

For both experiments, supplemental lighting was provided in the greenhouse to provide 16 h light and 8 h dark with Sun System III 1000-W metal halide lights (Sunlight Supply, Inc., Vancouver, WA) suspended from the ceiling. Artificial lights provided an average photosynthetically active radiation (PAR) of 1175 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the greenhouse. On a partly cloudy day, a typical ambient PAR (without lights) was 625 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Air temperature was maintained at 25 to 30°C with a heating system controlled by thermostat.

Chloroplast-transformed Petite Havana tobacco cultivar seeds for producing PA were provided by Dr. Henry Daniell. Anthrax PA tobacco seeds and Petite Havana (the original PA parent cultivar) seeds were planted in 253-cell Styrofoam float trays containing Premier Pro-Mix potting media (Riviere-du-Loup, QC, Canada) and floated in beds containing a modified Hoagland nutrient solution (Noggle and Fritz, 1976a).

Experiment 1: Transpiration with Potting Medium

When a tobacco is genetically engineered for a specific purpose such as producing disease vaccines, soil water use and response to irrigation by plants could be altered. Tobacco growth effects from chloroplast transformation vary depending on the classification and mode of action of the protein being produced. The most likely scenario for affecting soil water use by genetic engineering would be if the overall size of the plants was increased or decreased.

Leaf transpiration and photosynthesis of nontransformed Petite Havana and genetically modified plants in pots were monitored daily at the button stage (appearance of primary reproductive buds). Previous research had shown that the highest leaf PA levels occurred at button stage (M. Mulesky, 2005, unpublished data). To determine if the PA gene affected plant/water relations in the tobacco plants, daily water applications were stopped and the potting soil was allowed to become progressively drier.

A greenhouse experiment with pots was conducted from October to December 2006. A gravimetric procedure (also called the lysimeter method) was used to compare leaf transpiration rates at the button growth stage from PA tobacco plants and the original Petite Havana parent cultivar (Noggle and Fritz, 1976b). At the four-leaf stage, tobacco seedlings were transplanted from float trays to 800-cm³ plastic pots with no drain holes. Each pot contained 136 g (dry wt.) of Premier Pro-Mix potting media and 227 mL water. Seedlings from nontransgenic Petite Havana control and PA tobacco were arranged in a completely random design with six replications.

Additional plants of each tobacco line were grown for weekly cutting to measure plant biomass. Water was added to maintain the original potting medium water content to compensate for daily evapotranspiration. Nutrients were supplied to the tobacco by adding 0.5 g of 15–30–15 N–P–K (Scotts Miracle-Gro, Marysville, OH) per liter in the watering solution. The gravimetric experiment was begun when plants grew to the button stage.

To measure initial plant leaf area before beginning the experiment, each potted plant was placed in a 50- by 75-cm area on the floor and a camera mounted on a tripod was used to record an overhead digital image. SigmaScan Pro 5.0 software (Systat Software, Inc., Point Richmond, CA) and a batch processing macro (Purcell, 2000) were used to analyze each image for percentage of green pixels and to approximate the leaf area of the plants.

At the button growth stage, daily water applications stopped and transpiration monitoring began. Daily weights of all the plants with soil medium and pots were recorded until Petite Havana leaves were completely wilted and photosynthesis levels were near zero. By not watering plants, daily water use from potting soil became cumulative with time for PA and nontransgenic Petite Havana plants. Visual symptoms of plant water stress were recorded to indicate whether one group of plants wilted and died faster than the other group.

During the experiment, midday plant water use rate per unit of tobacco leaf area was also recorded each day. Transpiration rate from the youngest mature leaf (fourth from apex) of the six PA and nontransgenic plants was measured at 11 am to 1 pm using a LCpro+ portable photosynthesis system (ADC Bioscientific, Ltd., Hoddesdon, UK) equipped with a leaf chamber for measuring gas exchange from a 6.25-cm² leaf area (Dynamax Inc., 2006). The leaf chamber contained an internal light source, which was programmed for producing 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR to simulate full sunlight and a temperature control set at 25°C. The main console supplied greenhouse ambient air [420 vpm CO₂ (0.042%) and 1.7×10^{-3} mPa H₂O] to the leaf chamber. The air was directed over both surfaces of the leaf. From the differences in gas concentration and airflow rate, the assimilation and transpiration rates were calculated by the instrument every 20 s. When the leaf chamber was moved from one plant to the next, sufficient time was allowed for the readings to stabilize before recording transpiration and photosynthesis values. The instrument measured CO₂ by using an infrared gas analyzer and H₂O was measured by two humidity sensors.

Experiment 2: Irrigation with Native Soil

A greenhouse experiment with native soil was conducted from December 2004 to March 2005. Tobacco plants were transplanted into native soil in the greenhouse floor rather than growing plants in pots. This was done to simulate conditions in a large high tunnel greenhouse where tractors and other machinery are used to reduce production costs. In the midsouthern region of the USA, most greenhouse plant production is conducted during the fall, winter, and spring months to avoid the cooling problems associated with high summer temperatures.

Transgenic PA tobacco was grown in native soil to measure tobacco biomass and PA protein production with different amounts of irrigation. Tobacco seedlings were transplanted from float beds into the tilled earth floor, which was Tiptonville sandy loam soil (fine-loamy, mixed, thermic Typic Argiudolls) containing 1.0 g kg⁻¹ organic matter, 7.5 pH_{salt} (measured in 0.01 M CaCl₂), and 16.5 cmol_c kg⁻¹ cation exchange capacity. The available soil water holding capacity for this soil was be-

tween 0.50 to 0.63 cm water/cm soil (Brown, 1971). Soil tests showed that initial fertility levels, including N, were high in the greenhouse soil from previous cotton breeding research in the facility. University of Missouri soil test laboratory results for tobacco did not recommend any fertilizer. The soil was mechanically tilled and formed to make 120-cm-wide beds.

A randomized complete block design with four replications was used. All beds were saturated with irrigation water and allowed to drain 7 d before tobacco plants were transplanted. Tobacco seedlings were hand transplanted in 30- by 30-cm spacing in the greenhouse soil beds. Each plot (122 by 122 cm) contained 16 tobacco seedlings (Fig. 1). Watermark soil moisture sensors (Irrometer, Inc., Riverside, CA) were installed in the center of each plot with sensor elements buried 10 cm deep in the soil. Treatments consisted of four Φ irrigation threshold points (-20, -34, -48, and -62 kPa). Rainbird XS-090 sprinkler tips (Azusa, CA) mounted on plastic stakes were placed on opposite borders of each plot and adjusted to distribute irrigation water evenly without over-spraying adjoining plots. A shovel was used to construct narrow trenches and dikes between plots to prevent water runoff and inhibit encroachment of roots from border plants into neighboring plots. An electronic irrigation controller was used to turn on and off solenoids from a water supply pipe equipped with a 0.13 MPa pressure regulator. Soil water potential from the sensors was recorded each morning and evening. When the Φ dropped below a treatment threshold, irrigation water was applied in 0.5- to 1-cm applications. All plots received two posttransplant irrigations.

Tobacco plant measurements and harvest data were collected from the four center plants in each plot. Plant height and leaf width and length from the youngest fully developed leaves (located on fourth node from the apex) were measured each

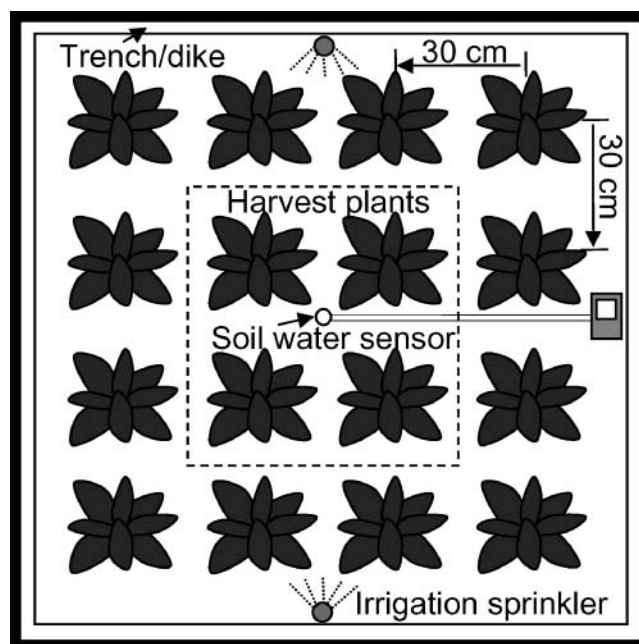


Fig. 1. Diagram of the arrangement of each plot for the irrigation experiment with soil in a glasshouse at Portageville, MO. Rainbird (Azusa, CA) XS-090 irrigation sprinklers were placed in opposite ends of each plot and adjusted to distribute water evenly without over-spraying plots. A narrow trench/dike was constructed around the border to contain run-off water and discourage root encroachment from neighboring plots. Watermark (Irrometer, Inc., Riverside, CA) soil moisture sensors were buried 10 cm deep in the center of each plot.

week. Five green color readings per leaf were collected weekly with a SPAD-502 chlorophyll meter (Minolta, Tokyo, Japan).

A ratoon system was employed with transgenic plants cut in the button stage and allowed to produce regrowth for a second harvest. Leaf PA yield in the tobacco was quantified by ELISA from the four irrigation treatments. Plants were cut 10 cm from the soil surface using horticulture hand pruners on 9 Feb. and 24 Mar. 2005. Leaf and stem material was separated and weighed. Results were reported on a fresh wt. basis because, unlike conventional tobacco production methodology, pharmaceutical-containing tobacco leaves are not dried before proteins are extracted. Approximately 3 cm² of tobacco leaf blade tissue was collected from each plant and placed into a 1.2-mL tube retained in a sampling box containing 96 individual tubes. Subsamples were frozen and tested for concentrations of PA.

ELISA Quantification of Protective Antigen

Leaf tissue samples (0.5–1.2 g), collected from transformed and untransformed plants, were ground in liquid N and re-suspended (2:1 diluent–tissue ratio) in a protein extraction solution (50 mM NaOH, 10 mM DTT, 5 mM EDTA, 1.5% PVP, pH 10). Anti-PA capture MAb (Bioscience Intl., Saco, ME) was diluted to 10 µg mL⁻¹ in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and bound to a 96-well microtiter plate (Immulon 2HB, Thermo Electron Corp., Walton, MA) overnight at 4°C. Wells were washed three times with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) before blocking (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min at room temperature. Recombinant PA (List Biological Laboratories, Inc., Campbell, CA) was diluted in buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0), to obtain a linear standard curve (0–0.8 µg mL⁻¹). Coomassie Plus Protein Assay reagent (Pierce Biotechnology, Inc., Rockford, IL) was used to determine treatment sample total soluble protein concentrations. Treatment samples were diluted in Tris-BSA-Tween buffer with 10 µg of protein loaded per well. Standards and samples were incubated at room temperature for 1 h. Plates were washed and incubated for 1 h with an anti-PA mouse monoclonal horseradish peroxidase (HRP) conjugated antibody (5.6 mg mL⁻¹; Bioscience International, Saco, ME), diluted 1:100,000 in sample-standard diluent. After washing, wells were incubated with tetramethylbenzidine (TMB) substrate for 30 min before the reaction was stopped by addition of 2 M H₂SO₄. Plates were read at 450 nm with a BP 800 microplate reader (BioHit Diagnostics, Neptune, NJ).

Tissue Nutrient Tests

Leaf samples were also digested for N, K, and P analyses using a modified wet acid digestion procedure (Mills and Jones, 1996). Tobacco leaf samples were dried at 100°C, ground, digested with a Hach Digesdahl Digestion Apparatus, 115Vac, 50/60 Hz (Hach Company, Loveland, CO) using H₂SO₄ and H₂O₂. Tissue SO₄-S was extracted from dried leaf samples by the acetic acid procedure (Eik, 1980). Leaf K content was tested with a PerkinElmer atomic absorption spectrophotometer (Wellesley, MA; Thomas, 1982). Phosphorus and N were tested colorimetrically (Laverty, 1963; Keeney and Nelson, 1982) and S was tested turbidimetrically (Rhoades, 1982) with a Genesys 10 spectrophotometer (Thermo Spectronic, Rochester, NY).

Statistical Analyses of Data

The statistical analyses of plant height, leaf size and nutrient content, biomass, and PA protein yield data were performed

using PROC MIXED v. 8.0 (SAS Institute Inc., Cary, NC). This procedure provided Type III *F* values but did not provide mean square values for each source of variation within the analysis or the error terms. Mean separation was evaluated through a series of pairwise contrasts among all treatments (Saxton, 1998). Probability levels > 0.05 were categorized as nonsignificant. Microsoft Office Excel 2000 (Microsoft Corporation, Redmond, WA) spreadsheet software was used to graph daily transpiration rates, calculate standard error, and develop regression equations from tobacco leaf SPAD chlorophyll meter readings.

RESULTS AND DISCUSSION

Experiment 1: Transpiration Experiments with Potting Medium

Significantly less vegetative growth was produced with PA tobacco as compared with the nontransgenic Petite Havana control. At the prebutton growth stage, fresh wt. of PA plants averaged 16 g (± 4 g) with 555 cm² (± 75 cm²) leaf area. Petite Havana plants weighed 37 g (± 5 g) with 629 cm² (± 54 cm²) leaf area. Soil water content and leaf transpiration rates both declined rapidly in the first 2 d after water was stopped, then slowly curved to near zero as plants wilted more hours each day (Fig. 2 and 3). Based on cumulative gravimetric soil water measurements, PA plants transpired water significantly slower from the potting medium than nontransgenic plants because of their smaller size (Fig. 2). Transpiration per unit leaf area in control and transgenic plants was similar in the first 2 d without watering (Fig. 3). However, after 6 d without watering, nontransgenic control plants were permanently wilted because of dry potting medium compared with PA plants that were moderately wilted at midday. Anthrax PA plants were probably not any more drought tolerant than control plants. Rather, because of their smaller leaf area, anthrax PA tobacco plants were able to continue photosynthesis at least 1 d longer because of more residual water in potting medium. This indicates that irrigation management may be different for antigen tobacco than nongenetically engineered tobacco. Because daily water use was less for PA tobacco,

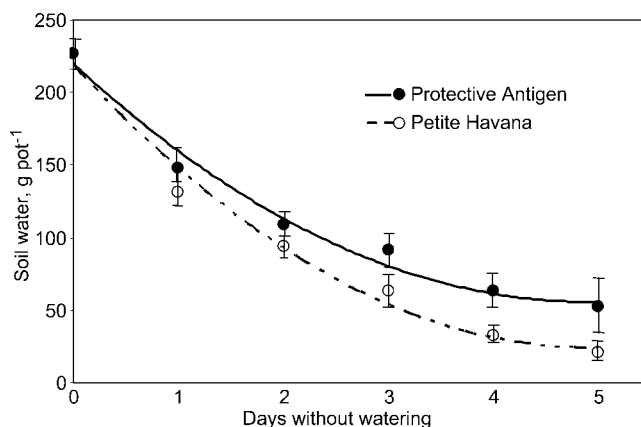


Fig. 2. Soil water weight loss from evapotranspiration during 5 d without watering from pots growing protective antigen tobacco and Petite Havana (nontransgenic parent) plants. Bars indicate standard error mean.

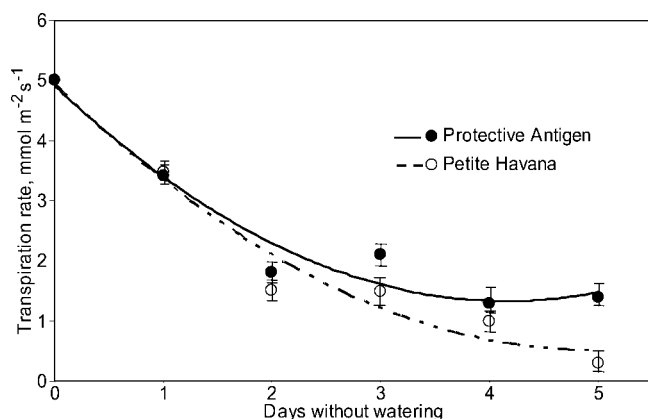


Fig. 3. Transpiration rate per leaf area during 5 d without watering from pots growing protective antigen tobacco and Petite Havana (nontransgenic parent) plants. Measurements were made with a portable photosynthesis system. Bars indicate standard error mean.

lower rates of water or longer intervals between irrigation applications may be needed.

Experiment 2: Irrigation with Native Soil

Anthrax PA tobacco plants grew well when planted directly in the alluvial soil of the greenhouse floor (visual observation). After the transplants were established, a typical irrigation schedule was 1 cm water every other day for the -20 kPa treatment, 1 cm water every third day for the -34 kPa treatment, 1 cm water every fourth day for the -48 kPa treatment, and 1 cm water every fifth day for the -62 kPa treatment.

Harvest cuttings of PA tobacco plants occurred on 9 Feb. and 24 Mar. 2005. After the first cutting, multiple shoots sprouted from the main stems. This helped fill in the gaps between plants and increase light interception. Tobacco plants on the second harvest cutting averaged 17 cm taller ($P < 0.05$) than plants on the first harvest date (data not shown). Date of harvest did not significantly interact with irrigation treatments for plant height, leaf measurements, N, P, K, and S leaf content, or biomass yields and dates were pooled for analysis.

Frequency of irrigation had an effect on the intensity of green color in leaves. Anthrax PA leaves in the driest irrigation treatments (-48 and -62 kPa Φ) were the darkest green based on Minolta SPAD chlorophyll readings (Fig. 4). Data indicate that chloroplasts may be less dense in PA leaves that received suffi-

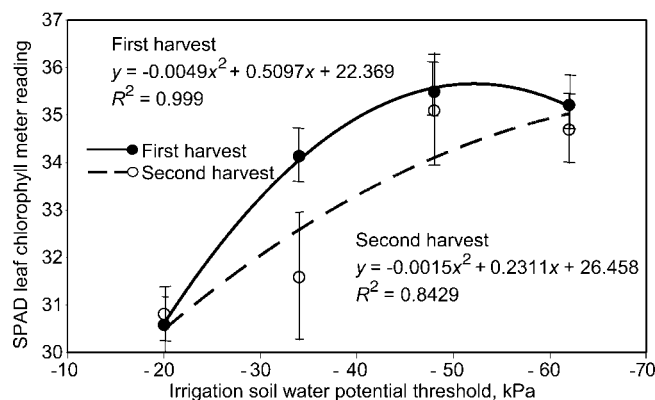


Fig. 4. SPAD chlorophyll meter readings from youngest developed leaf (fourth from apex) of protective antigen tobacco as affected by irrigation threshold based on soil water potential. Leaf measurements were made before both harvests. Points are means from four replicated plots with tobacco grown on Tiptonville sandy loam soil. Bars indicate standard error mean.

cient water to maximize biomass growth. This was a concern because PA is produced in the chloroplasts. Averaged across harvest dates, the driest irrigation treatment (-62 kPa) resulted in significantly shorter main stem height than other irrigation treatments (Table 1). This indicated that water stress caused a more compact plant with less potential for dilution of chemicals in the tissues.

No tissue dilution effect was found for N, P, and K nutrients in tobacco leaves from irrigation (Table 1). Tissue P and K content analyses showed that each nutrient was present above critical sufficiency levels in tobacco leaves at the button stage of growth (Mills and Jones, 1996). Sifola and Postiglione (2003) found that late-season irrigation increased conventional tobacco N uptake rate. In our study, irrigation did not significantly affect leaf N or S content, which are often associated with green color intensity in plant leaves. Based on deficiency ranges in the literature, both nutrients exceeded sufficiency levels at the prebutton growth stage (Mills and Jones, 1996). Since N and S content were not factors, the pale green or yellow leaf color in the -20 kPa irrigation threshold treatment may have been caused by depleted soil oxygen for roots from waterlogged soil.

In conventional tobacco production, Orphanos and Metochis (1985) found that the magnitude of yield increase from irrigation was greater for fresh wt. than dry

Table 1. Main stem height and youngest fully developed leaf length, width, and N, P, K, and S contents of PA tobacco averaged across harvests (9 Feb. and 24 Mar. 2005) as affected by irrigation soil water potential (Φ) threshold grown on Tiptonville sandy loam soil in a greenhouse at Portageville, MO.

Irrigation Φ threshold kPa	Main stem height† cm	Fourth node leaf from apex†					
		Length	Width	N	P	K	S
				g kg ⁻¹			μg kg ⁻¹
-20	67 a‡	45 a	21 a	49 a	3.0 a	67 a	1670 a
-34	67 a	45 a	22 a	44 a	3.0 a	66 a	1569 a
-48	66 a	43 a	19 a	50 a	2.9 a	66 a	1553 a
-62	58 b	41 a	19 a	47 a	2.7 a	65 a	1754 a

† Fourth node leaf from apex was the youngest fully developed leaf on tobacco plants.

‡ Main stem height, leaf length, width, N, P, K and S values followed by the same letter were not significantly different at the 0.05 probability level. Mean separation was evaluated through a series of pairwise contrasts among all treatments.

Table 2. Stem and leaf fresh weights of protective antigen vaccine tobacco harvested on 9 Feb. and 24 Mar. 2005 as affected by irrigation soil water potential (Φ) threshold on a Tiptonville sandy loam soil in a greenhouse at Portageville, MO.

Irrigation Φ threshold	First harvest, 9 Feb.†			Second harvest, 24 Mar.			Mean		
	Stem	Leaf	Total	Stem	Leaf	Total	Stem	Leaf	Total
kPa	— kg fresh wt. ha ⁻¹								
–20	6960 a	9132 a	16092 a	7554 b	9313 b	16867 b	7257 a	9223 b	16480 b
–34	6533 a	9475 a	16008 a	11262 a	13818 a	25080 a	8898 a	11647 a	20545 a
–48	3606 b	5817 b	9423 b	7169 b	8595 b	15764 b	5388 b	7206 c	12594 c
–62	3378 b	5573 b	8950 b	5604 b	8302 b	13906 b	4491 b	6938 c	11429 c

† Within a column, biomass weights followed by the same letter were not significantly different at the 0.05 probability level. Mean separation was evaluated through a series of pairwise contrasts among all treatments.

wt. In our study, irrigation significantly affected PMP tobacco stem and leaf fresh weights and PA content in the tobacco leaves (Tables 2 and 3). The highest leaf weight yields were found in the –34 kPa irrigation treatment on both harvests, although values were not significantly different between –34 and –20 kPa for the first harvest. Total biomass yield from the –34 kPa treatment was almost double the amount from the –48 and –62 kPa treatments.

The main factor effect for harvest date on leaf PA content was not significant. The most important information learned from this study was that optimizing irrigation management to increase biomass production did not dilute the PA concentration in the tobacco leaves. This was unexpected since the tobacco leaves were darker green (higher chlorophyll readings) in the driest irrigation treatments. The –34 kPa Φ threshold irrigation treatment, which produced the highest leaf fresh weight yield, also produced the highest PA concentration in leaves (Table 3). A significant interaction was not found between irrigation and harvest date main effects. Averaged across harvest dates, PA content for the –34 kPa Φ threshold was significantly higher than the wetter –20 kPa Φ treatment but not significantly different from drier treatments. Analyzed separately for each harvest date, the PA differences between irrigation treatments were not significant at the 0.05 level on the first harvest date. However, ANOVA showed a probability $> F$ of 0.09 for irrigation effect on leaf PA content. At the second harvest, irrigation with the –20 kPa threshold on average reduced PA 89% as compared with the –34 kPa irrigation threshold treatment (48 vs. 423 $\mu\text{g PA kg}^{-1}$ fresh leaf weight). Total soluble protein levels in leaves were not significantly impacted by irrigation.

For tobacco vaccine production in greenhouse soil, the second harvest may be more valuable because of the

higher leaf fresh weight yields and greater consistency in leaf PA content. At the first harvest, mean PA levels for irrigation treatments had a coefficient of variation of 49%. At the second harvest, more time had elapsed since plants had been transplanted. This extra time for root establishment helped produce more uniform plant growth and budding initiation. Timing harvest is critical because PA is highest at button stage. At first harvest, slight differences in early reproductive development between tobacco plants probably contributed to the erratic leaf PA contents between replications.

Although tobacco stems are usually included with the leaves in the initial processing after harvest, PA content in stems is very low (results not shown). In screw press processing, stems are often included with leaves mainly to help push tobacco leaves physically through the system.

SUMMARY

Key factors affecting the profitability of PMP manufacturing of vaccines are market value of the drug or vaccine, expression level of the target protein in plant tissue (leaves or seeds), and yield of tissue expressing the protein. Extracting and purifying proteins from dilute concentrations in tissue is very costly for companies that genetically engineer crops to produce pharmaceuticals. Optimizing agronomic parameters that can impact biomass and antigen expression is critical for cost-efficient production.

We found no evidence that providing optimum irrigation to PA tobacco plants resulted in diluted antigen levels in leaf tissue. The dilution effect that sometimes occurs in leaf tissue nutrients after rapid plant growth response from irrigation was not observed for PA content. Irrigating pharmaceutical tobacco too frequently was detrimental to antigen leaf concentration.

Table 3. Protective antigen in tobacco leaves on 9 Feb. and 24 Mar. 2005 as affected by irrigation soil water potential (Φ) threshold grown on Tiptonville sandy loam soil in a greenhouse at Portageville, MO.

Irrigation Φ threshold	Protein in tobacco leaves, fresh weight basis†					
	First cut, 9 Feb.		Second cut, 24 Mar.		Mean	
	Total soluble	Protective antigen	Total soluble	Protective antigen	Total soluble	Protective antigen
kPa	mg g ⁻¹	$\mu\text{g kg}^{-1}$	mg g ⁻¹	$\mu\text{g kg}^{-1}$	mg g ⁻¹	$\mu\text{g kg}^{-1}$
–20	7.6 a	277 a	9.2 a	48 b	8.4 a	163 b
–34	6.4 a	635 a	10.8 a	423 a	8.6 a	529 a
–48	5.6 a	272 a	9.6 a	173 b	7.6 a	223 ab
–62	6.9 a	419 a	10.4 a	103 b	8.6 a	261 ab

† Within column, total soluble protein or leaf protective antigen contents in columns followed by the same letter were not significantly different at the 0.05 probability level.

Chloroplast transformation for antigen production had no significant effect on transpiration rate per unit leaf area when adequate soil moisture was provided. However, Petite Havana plants (the parent cultivar) of the same age were larger than PA plants. This resulted in a higher daily water use rate in nontransformed plants. The implication of slower soil water use by PA tobacco to irrigation scheduling is that the optimum interval between irrigation applications may be longer for PA tobacco.

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